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Thermoregulation of *N*-Acyl Homoserine Lactone-Based Quorum Sensing in the Soft Rot Bacterium *Pectobacterium atrosepticum*▽

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The psychrotolerant bacterium *Pectobacterium atrosepticum* produces four *N*-acyl homoserine lactones under a wide range of temperatures. Their thermoregulation differs from that of the exoenzyme production, described as being under quorum-sensing control. A mechanism involved in this thermoregulation consists of controlling *N*-acyl homoserine lactones synthase production at a transcriptional level.

Several gram-negative bacteria synthesize *N*-acyl homoserine lactone (HSL) signal molecules that serve for cell-to-cell communication called for the first time quorum sensing (QS) by Fuqua et al. (7). Such regulatory systems operate to allow bacteria to sense cell density and to synchronize the functions of the entire population (reviewed in references 15, 23, and 29). In the *Pectobacterium atrosepticum* species (formerly *Erwinia carotovora* subsp. *atroseptica*) (8), QS regulation is involved in pectinolytic, cellulase, and protease activities, tuber maceration, and harpin synthesis leading to a hypersensitive response in nonhost plant (18, 25). In contrast, QS via HSL production does not regulate growth and mobility during initial infection events or during liquid culture in a synthetic medium (25).

Pectobacterium atrosepticum is a psychrotrophic bacterium involved in the soft rot of *Solanum tuberosum* (17, 26). Consequently, it causes important losses within cool temperate regions, where potatoes have traditionally been grown. The optimal temperature for pathogenicity, estimated to be around 20°C (19, 21), is a good compromise allowing both a fast multiplication (optimal at 24°C) and an efficient production of lytic enzymes, which is optimal at temperatures ranging between 12 and 24°C (26). As the thermoregulation of bacterial multiplication differs from that of exoenzyme production, we investigated the thermoregulation of QS that triggers exoenzyme synthesis. Therefore, HSL production and accumulation were measured during the three growth phases of a typical *P. atrosepticum* strain (Table 1) cultured at six temperatures. The role of the *expI* gene encoding the HSL synthase was determined by cloning this gene in *Escherichia coli* and by engineering a mutated strain of *P. atrosepticum*. Finally, the transcription of *expI* was estimated by relative reverse transcription-PCR (RT-PCR) under the same range of temperatures.

Effect of temperature on *N*-acyl HSL diversity and levels. Bacterial cultures were grown at 8, 12, 15, 20, 24, and 28°C in minimal medium with polygalacturonic acid (PGA) as the sole source of carbon. This vegetable compound induces synthesis of virulence factors involved in plant disease or resistance that have been demonstrated to be under QS control (25). Characterization of HSLs was performed by high-performance liquid chromatography coupled with mass spectrometry (16). In these conditions, *P. atrosepticum* 6276 produces four HSLs: mainly *N*-3-oxo-octanoyl-L-HSL (3-oxo-C8-HSL) and minor quantities of *N*-octanoyl-L-HSL (C8-HSL), *N*-3-oxo-hexanoyl-L-HSL (3-oxo-C6-HSL), and *N*-3-oxo-decanoyl-L-HSL (3-oxo-C10-HSL). The HSL levels increase from the middle to the end of the logarithmic phase and then decline during the stationary phase (Fig. 1). Growth temperature significantly influences HSL quantities produced, and optimal HSL production occurs at 24°C. In this psychrotolerant species, significant HSL levels were measured at 12°C and HSL production was still observed at 8°C. In contrast, a negative effect appeared at a warmer temperature (28°C). Lastly, the four HSLs were produced and accumulated in the same way at each temperature (Fig. 1). These data do not allow an association of the production of a particular HSL with a typical growth phase or temperature.

Cloning, sequencing, and role of *expI*. The primers ExpI1C (5′TGAATTGGGCGGTAAAAATGT3′) and ExpI2C (5′AATTCACCGTTGCCAAGAAG3′), deduced from the *E. carotovora* subsp. *carotovora* SCC1 genome (22), were used for amplification of *expI* by PCR. The single PCR product (651 bp) was cloned into the pMOS *Blue* vector as described by the manufacturer (pMOS *Blue* blunt-ended cloning kit; Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) and introduced into *E. coli* XL1-Blue cells by electroporation according to standard procedures (13). Positive clones were checked by PCR using the two above-described oligonucleotides as primers, and the sequence of the cloned fragment was determined (Genome Express, Meylan, France). In silico sequence analyses were performed with programs available at the Infobiogen web site (Bioinformatic Resources center; http://www.infobiogen.fr/). A unique gene, named *expI* for *ex*oenzyme *p*roduction *i*nducer (20), was identified in *P. atrosepticum* 6276 (GenBank accession no. AJ580599). Comparison of sequences of the 216-amino-acid-residue peptide deduced from in silico translation showed, respectively, 98% and 97% identities with *E. carotovora* subsp. *caro-*

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TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Relevant characteristic(s)	Reference(s) or source
Bacterial strains		
Pectobacterium atrosepticum (*Erwinia carotovora* subsp. *atroseptica*)		
CFBP 6276	Potato soft rot pathogen, HSL producer	26
6276-EI	*expI* mutant derivative of CFBP 6276 strain	This work
Escherichia coli		
DH5αmcr	F′ ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_{K12}^- m_{K12}^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	24
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17*(r_{K12}^- m_{K12}^+) *supE44 relA1 lac* [F′ *proA*$^+$*B*$^+$ *lacI*^{q}*Z*Δ*M15*: Tn*10* (Tc^r)]	12
S17-1λ*pir*	*pro thi hsdR*$^-$*M*$^+$ *recA* RP4:2Tc:Mu:KmTn*7* Sm^r Tp^r phage λ_{pir} lysogen	5, 29a
Plasmids		
pMOS*Blue*	Broad-host-range cloning vector, 2,887 bp; Ap^r	24
pMOS*Blue*/*expI*	pMOS*Blue* carrying the *P. atrosepticum* 6276 *expI* gene; Ap^r	This work
pAG408	Vector containing *atpE-gfp*$_{uv}$ minitransposon, 5,700 bp; Ap^r Km^r Gm^r	27

[a] Ap^r, Gm^r, Km^r, Sm^r, Tc^r, and Tp^r indicate resistance to ampicillin, gentamicin, kanamycin, streptomycin, tetracycline, and trimethoprim, respectively; CFBP, Collection Française de Bactéries Phytopathogènes, Institut National de la Recherche Agronomique, Angers, France.

tovora EC153 and *E. carotovora* SCC3193 HSL synthase proteins (4, 20).

An HSL-deficient *P. atrosepticum* 6276 mutant was obtained by random transposon mutagenesis. A bank of random insertion mutants was made by using the mini-Tn*5* transposon delivery system carried by the pAG408 suicide vector (27). The *Escherichia coli* S17-1λ*pir* strain was used as the donor in a mating experiment to transfer the suicide plasmid into *P. atrosepticum* 6276. Transconjugants were selected on PGA agar (25) plates supplemented with kanamycin (50 $\mu g \cdot liter^{-1}$) and gentamicin (30 $\mu g \cdot liter^{-1}$) and then tested on indicative LB plates containing an HSL monitor bacterium (*Chromobacterium violaceum* CV026) (14). Among transconjugants analyzed, one did not display purple color in the surrounding agar. For this mutant strain, named *P. atrosepticum* 6276-EI, PCR experiments show that the transposable element is integrated approximately 500 bp downstream from the *expI* translation start codon.

At the optimal temperature of production (24°C), HSL synthesis was totally altered in *P. atrosepticum* 6276-EI, since no HSL was detected. In contrast, tenuous but quantifiable amounts of 3-oxo-C8-HSL and C8-HSL were detected in the *E. coli* XL1-Blue strain harboring the complete *expI* gene when grown at 24°C in LB medium, but no HSLs were detected in the wild strain (data not shown). Soft rot in tuber was assessed by testing at the optimal temperature of HSL production as described previously (25). The test confirmed, in planta, the importance of the *expI* gene in the QS and in the consequent tissue maceration. No macerated tissue was found on tubers 7 days after inoculation with *P. atrosepticum* 6276-EI (Fig. 2). This lack of maceration is attributed to the absence of HSL synthesis in the potato tuber.

Effect of temperature on *expI* expression. The kinetics of the *expI* transcription were determined by semiquantitative RT-PCR experiments with *P. atrosepticum* 6276 cultured in PGA medium. Total RNAs were extracted as described by the manufacturer (RNA isolation kit; Roche Diagnostics, Mannheim, Germany). For calibration, an RT-PCR was also performed using R16S1C (5′-GGGGGTAGAATTCCAGGTGT-3′) and R16S2C (5′-CGGCAGTCTCCTTTGAGTTC-3′) primers, amplifying a unique 495-bp fragment of the *P. atrosepticum* E1 16S ribosomal gene (6). At each studied temperature, *expI* transcript amounts were most important at the end of the exponential growth phase. From 8°C to 24°C, amounts of transcripts increased in the same manner as the growth temperature, and a negative effect appeared at 28°C (Fig. 3). The thermoregulation of *expI* transcription and that of HSL levels are identical throughout the bacterial culture (Fig. 1 and 3).

Pectobacterium atrosepticum 6276 harbors the QS signaling system of class I recently defined by Chatterjee et al. (4). This trait is linked to the structural characteristics of its HSL synthase (2, 28). We suggest that only 3-oxo-C8-HSL corresponds to a QS signal and that the others are less specific products of ExpI synthase or catabolites that appear during HSL turnover. This hypothesis is supported by (i) HSL concentrations usually measured in other *Pectobacterium* strains (1, 3, 30), (ii) the absence of HSL synthesis in an *expI*-deficient mutant, (iii) the major production of this HSL by another bacterial species (*E. coli*) complemented with *expI*, and (iv) the identical thermoregulation of each HSL.

The effect of temperature on extracellular HSL concentration is the concomitant result of HSL production and degradation. Previous studies showed that HSL degradation could be influenced by pH or temperature: nonenzymatic lysis is increasingly favored in LB medium with increasing pH (from neutral to mildly alkaline) and temperature over a range from 22 to 37°C (3, 31). However, in PGA medium, during the bacterial growth, a decrease of pH from 8 to 7.2 is observed (data not shown). In these conditions, the HSL autolysis seems to be minor, since we observed at each temperature tested an increase of HSL production from the log exponential to the early stationary phase over a decrease in the pH of the medium (Fig. 1). Here we show that the thermoregulation of QS molecules reflects the thermoregulation of HSL synthesis.

Another report in this work is that the QS thermoregulation is identical to that of the bacterial growth that we have previ-

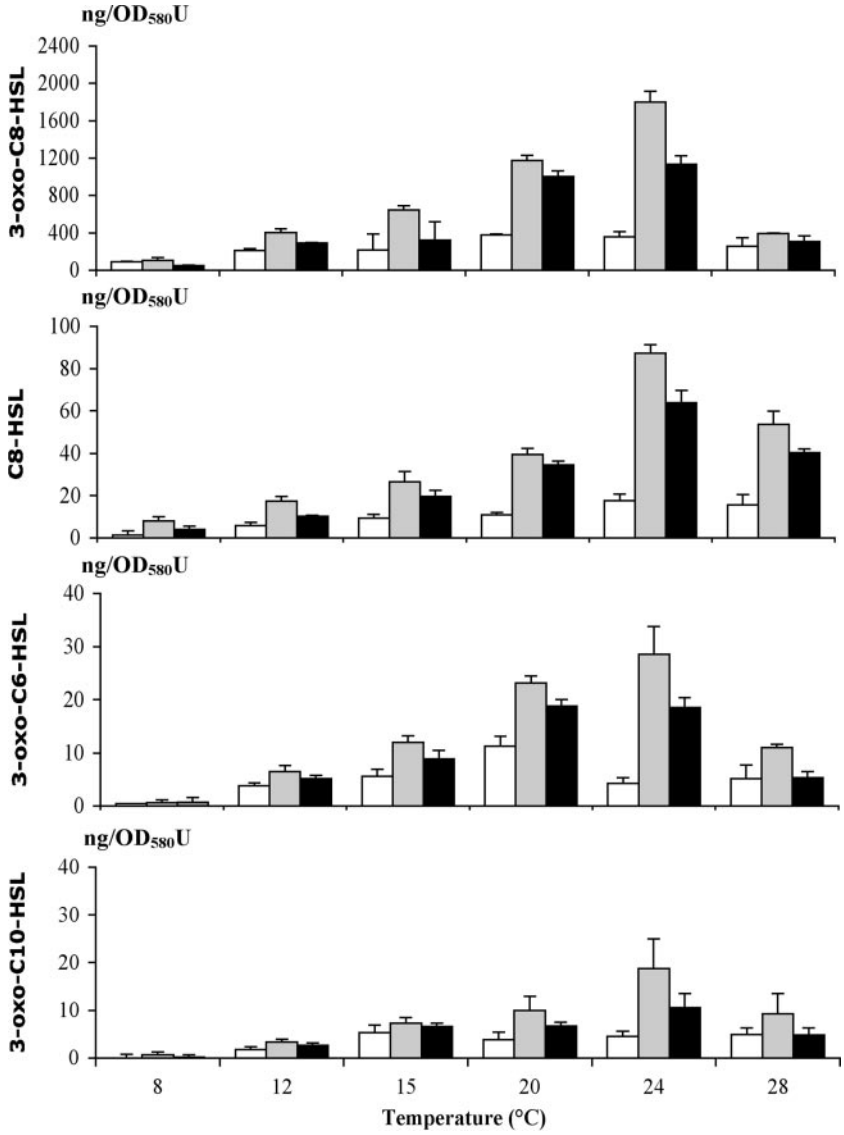

FIG. 1. Effect of temperature on HSL production and accumulation by *P. atrosepticum* 6276. The HSLs were extracted from supernatant at exponential phase (white columns), late exponential phase (gray columns), and stationary growth phase (black columns). For each temperature point, at least three independent cultures were made. Each bar represents the standard deviation. $OD_{580}U$, unit of optical density at 580 nm.

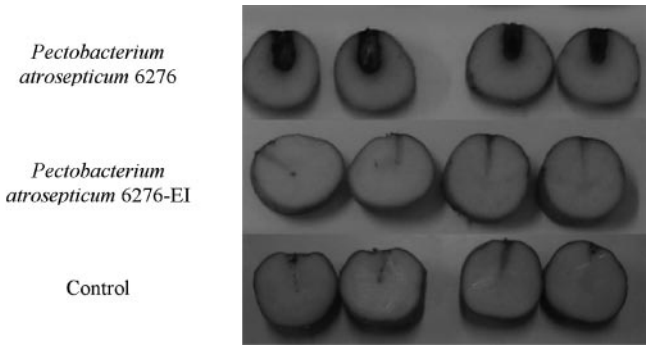

FIG. 2. Role of *expI* gene on tuber disease. Potato tubers were infected by *P. atrosepticum* 6276 and incubated at 24°C. The development of symptoms was evaluated after 7 days.

ously described for strain 6276 under the same culture conditions (26). However, by engineering *P. atrosepticum* 6276 which expresses a lactonase and so is unable to produce high concentrations of HSLs, we have shown that QS does not control bacterial growth in vitro (PGA medium) and in planta (25). This suggests that the HSL synthesis pathway is not thermoregulated differently from the main pathways involved in the basal metabolism. Besides, our results show that one of the mechanisms involved in the QS thermoregulation consists of controlling HSL synthase production by modulating the amounts of *expI* transcripts. This temperature-dependent regulation could occur at the level of transcription initiation involving temperature-sensitive sigma factors or by production of small RNAs which would act as thermosensors (9, 10). In contrast, the QS thermoregulation differs from that one of the main virulence factors studied in the soft rot bacterium *Pectobacterium*. Indeed,

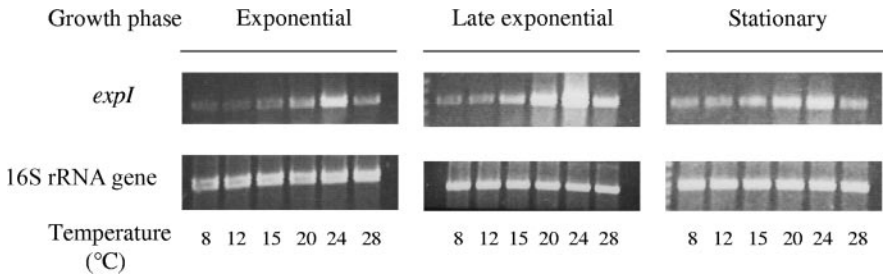

FIG. 3. Effect of temperature on *expI* gene expression in *P. atrosepticum* 6276. Transcription of *expI* was determined by RT-PCR experiments with cells harvested from exponential phase, late exponential phase, and stationary growth phase. Results obtained on 1% agarose gels were expressed as the ratio of *expI* transcripts to 16S transcripts.

these bacteria secrete a large variety of extracellular lytic enzymes. Among them, the pectate lyase has been described as the most important cause of maceration and cell killing (11). In *P. atrosepticum* 6276, the optimal temperature for pectate lyase production (between 12 and 15°C) is lower than the optimal temperature for HSL synthesis (24°C), while this exoenzyme production is under QS control (25, 26). These results strongly suggest that the thermoregulation of pectate lyase production occurs downstream of quorum signaling.

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